



Regulation of CD44 hyaluronan receptor function by protein acylation

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Warren received his Ph.D. in 1981 from the Department of Biochemistry at the University of Illinois in Urbana Champaign. From 1981 to 1985 he was a postdoctoral fellow with

Bryan Toole at Tufts University in Boston. In 1985 he joined Klaus Kuettner and the faculty at Rush Medical College. In 2000 he became Professor of Biochemistry at Rush and in 2003 was named the Ralph and Marion C. Falk Endowed Chair Professor of Biochemistry. In March of 2006, after 21 years in Chicago, Warren Knudson moved to the Department of Anatomy and Cell Biology at the Brody School of

Medicine at East Carolina University in Greenville, North Carolina. Dr. W. Knudson's area of interest concerns the overall metabolism of hyaluronan. His laboratory has explored the expression and function of hyaluronan synthases, hyaluronidases and the hyaluronan receptor CD44 in articular chondrocytes and a variety of cell lines. His work is aimed at addressing changes in hyaluronan metabolism associated with osteoarthritis. The current focus concerns the internalization and degradation of hyaluronan via CD44, mechanisms associated with CD44 signaling, and lysosomal hyaluronidase expression. Dr. W. Knudson works closely with Dr. Cheryl B. Knudson especially in the area of CD44 and cell-matrix interactions.

Fig.1A

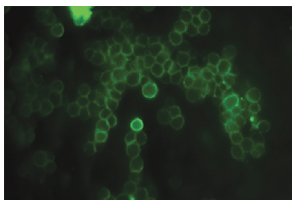


Fig.1B

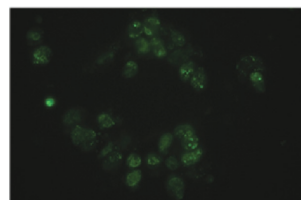
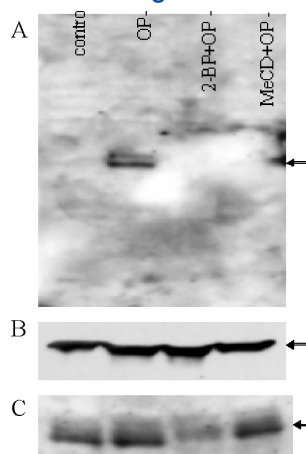


Fig.1. FITC-cholera toxin B subunit staining of bovine articular chondrocytes before (Fig.1A) and after (Fig.1B) pretreatment with methyl-beta cyclodextrin (MeCD).

Fig.2

Fig.2. Western blot of chondrocyte lysates following OP-1 treatment with or without pre-incubation with 2-bromopalmitate (2-BP) or MeCD. Arrow in panel A depicts migration position of phospho-Smad-1; panel B arrow, total B-actin and; panel C arrow, total Smad-1.



The interaction between hyaluronan and cell surfaces is an important means of communication between the extracellular matrix and cells. One issue that remains to be resolved is how cells regulate the residency time of hyaluronan at the cell surface. CD44 is the principal cell surface receptor for hyaluronan on many cell types. In addition, CD44 mediates the endocytosis of hyaluronan leading to its subsequent degradation within lysosomes. Thus, one mechanism cells may use to regulate the turnover of cell surface hyaluronan is by regulating CD44-mediated internalization. However, the nature of this regulation is unknown as CD44-mediated internalization of hyaluronan appears independent of classical clathrin-coated vesicles, caveolin or pinocytosis pathways. Using model cell systems, we observed that depletion of membrane cholesterol

with methyl-beta cyclodextrin had no effect on the cell surface binding of hyaluronan but, completely blocked its internalization. This suggested that hyaluronan internalization required the presence of lipid raft microdomains. Next, we demonstrated that a pool of CD44 resides in low density membrane fractions, indicative of lipid rafts. The association of CD44 with these low density fractions could be inhibited by depletion of cholesterol or pre-treating the cells with an inhibitor of protein palmitoylation, 2-bromo-palmitate. This suggested that a fraction of membrane-associated CD44 was acylated and that acylation was required for CD44 association with lipid rafts. Further, we demonstrated that palmitoylation of CD44 on two highly conserved cysteine residues is essential for the association with lipid rafts as determined by density gradient ultracentrifugation. Mutations of either cysteine residue (cys²⁸⁶ or cys²⁹⁵) or pre-treatment of cells with the palmitic acid analog 2-bromopalmitate, reduced the [³H]-palmitic acid incorporation into CD44 and prevented CD44-lipid raft association. Preventing CD44 palmitoylation had no effect on the binding of hyaluronan to COS-7 transfectants or chondrocytes but inhibited hyaluronan internalization. The turnover of the CD44 receptor itself was also affected by blocking its association with lipid rafts. Using cycloheximide to prevent de novo protein synthesis, palmitoylation deficient cysteine CD44 mutants underwent slower turnover from cell surface compared to the palmitoylation intact wild type CD44, as determined by immunofluorescence and western blotting. These results indicate that palmitoylation of CD44 is a critical driving determinant to CD44 association with lipid rafts, and concomitantly the rates of hyaluronan endocytosis and CD44 turnover from cell surface. Additional preliminary evidence suggests that the expression of a thio-esterase may, in turn, regulate the degree of CD44 palmitoylation. The next question to be asked

is whether the association of CD44 with lipid raft microdomains has regulatory effects on other CD44-related functions such as cell signaling, the role of CD44 as an anchoring co-receptor or, the shedding/release of CD44 and its intracellular domain. One example of a dual signaling and co-receptor function of CD44 occurs in chondrocytes where CD44 has been shown to influence signaling events induced by bone morphogenic protein-7 (BMP-7, aka OP-1). A yeast two-hybrid screen identified an interaction between the cytoplasmic tail of CD44 and Smad-1. Subsequent immunoprecipitation assays confirmed this interaction and truncated CD44 dominant negatives blocked Smad-1 / Smad-4 nuclear translocation following treatment with BMP-7. The next question was whether chondrocytes also exhibit lipid raft microdomains and if so, were they important for CD44 / Smad-1 interactions. In current studies we document that chondrocytes stain with a FITC-cholera toxin subunit probe—a probe that binds GM1 gangliosides that are enriched within lipid rafts. The FITC-cholera toxin B subunit appears evenly distributed (Figure 1A) around the chondrocyte plasma membrane. Upon depletion of cholesterol, the toxin localization becomes sparse, punctuate and seemingly intracellular (Figure 1B). Cholesterol depletion also blocks CD44 association with low density membrane fractions. Interesting, the depletion of cholesterol with methyl-beta cyclodextrin or, interference of protein palmitoylation with 2-bromopalmitate, both blocked the phosphorylation of Smad-1 in chondrocytes in response to BMP-7 (Figure 2). Additional studies will be required before definitive conclusions can be made. However, it is likely that the involvement of protein palmitoylation and lipid raft association of receptors will serve as an important method for cellular regulation of multi-functional receptors such as CD44.

Keywords: CD44, hyaluronan, BMP-7, Smad-1, lipid rafts, endocytosis